Supplementary data:

Figure 1:

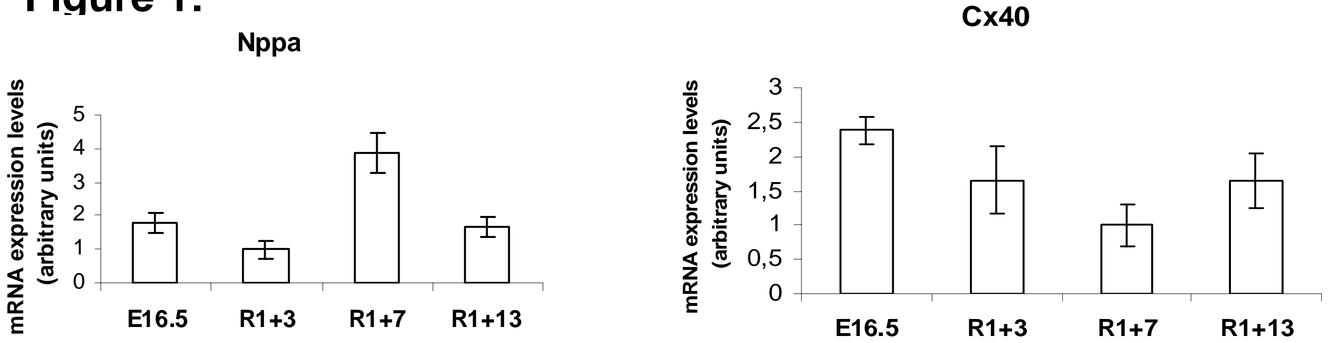


Figure 2:

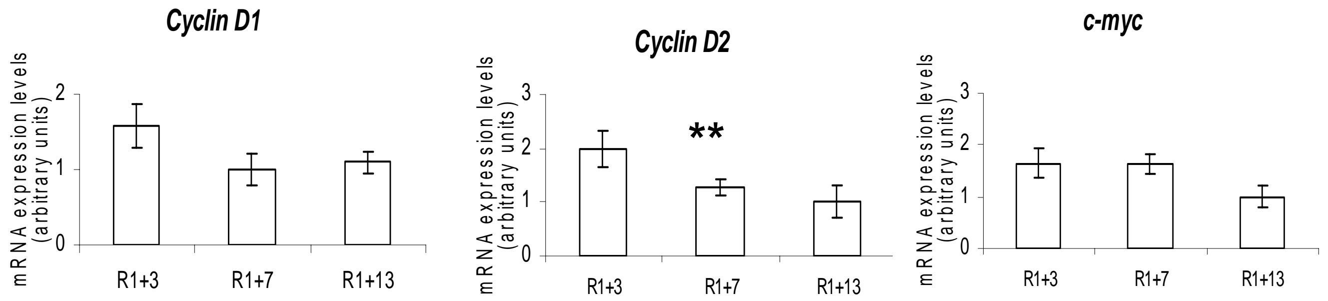


Figure 3:

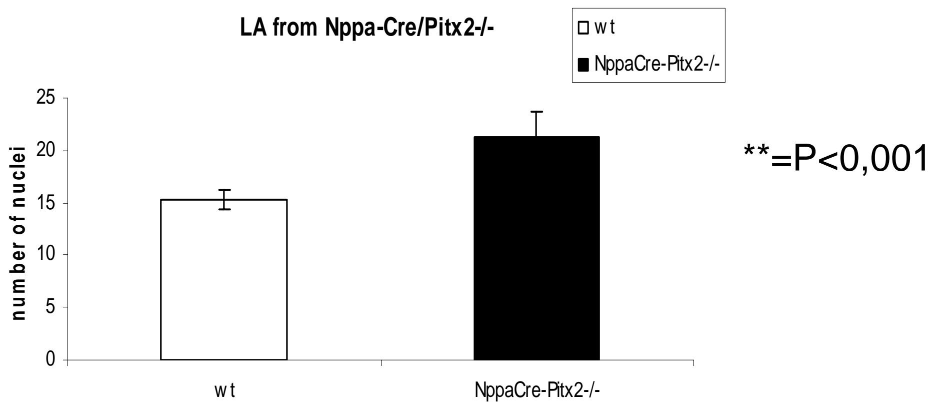
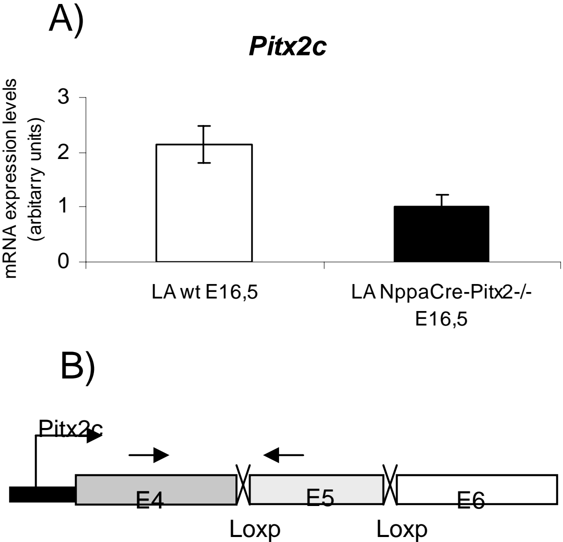
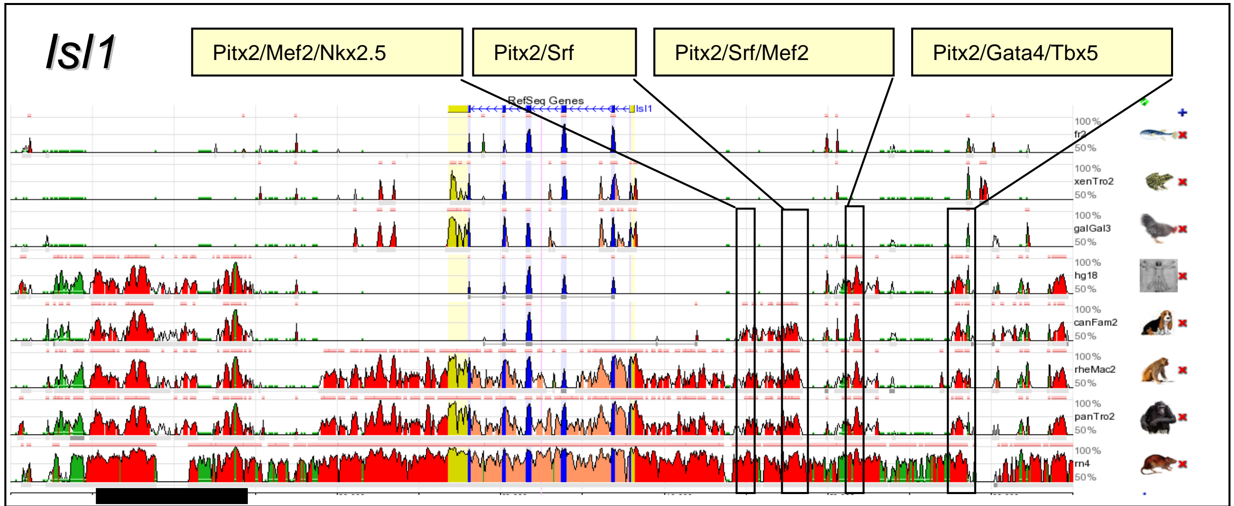


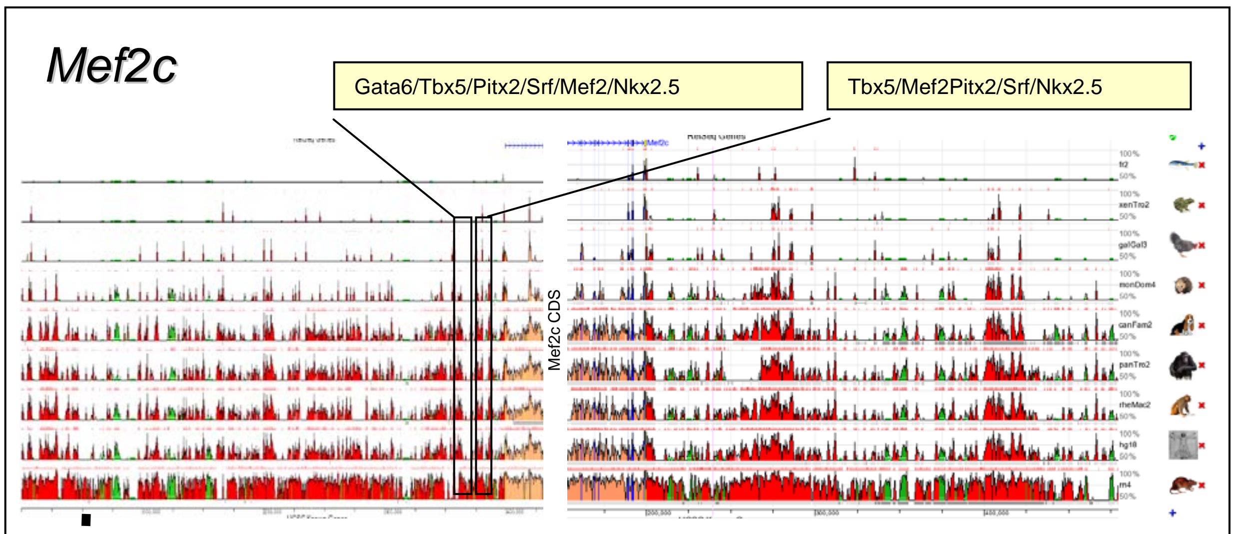
Figure 4:

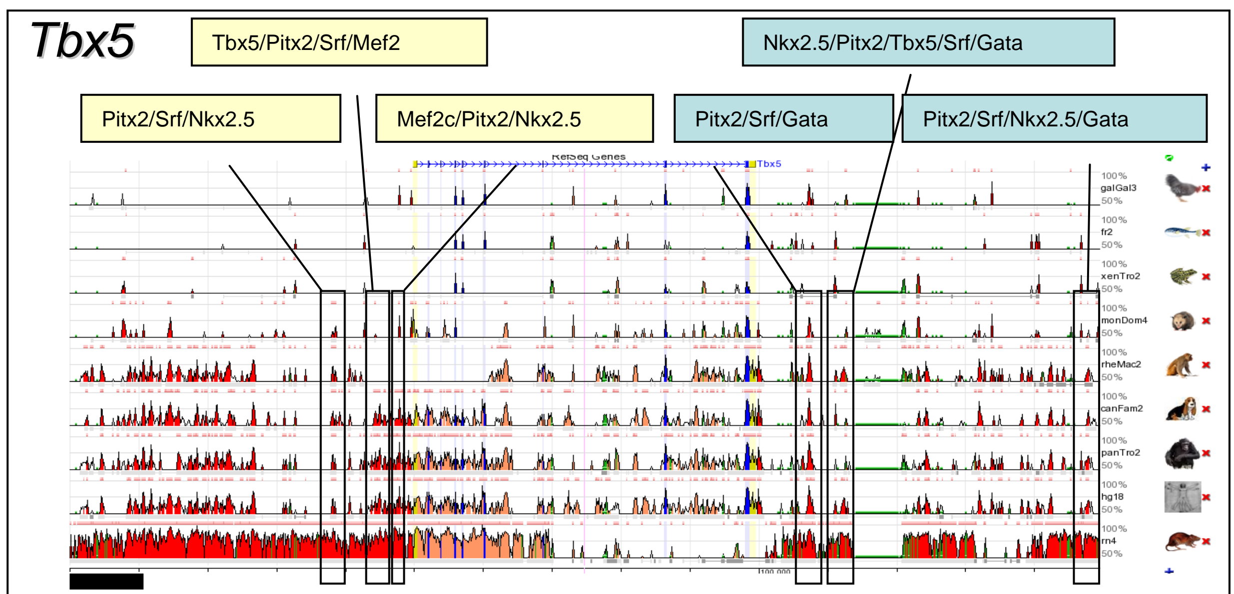


Supplementary data:

Figure 5:







SUPPLEMENTARY DATA: FIGURE LEGENDS

Figure 1: Comparative analyzes for the expression levels of *Nppa* (A) and *Cx40* (B) genes in native tissue (mouse embryonic heart at E16.5) and beating areas. Note that the mRNA expression levels of *Nppa* are equivalent between native tissue and beating areas obtained at early (R1+3 and late (R1+13) stages of *in vitro* differentiation.

Figure 2: Quantitative RT-PCR to analyze gene expression profiles for cell cycle genes *Cyclin D1*, *Cyclin D2* and *c-myc* in beating areas obtained from R1-ESC at early (R1+3), intermediate (R1+7) and late (R1+13) stages of *in vitro* differentiation.

Figure 3: Confocal images were obtained from distinct regions of the developing atrial appendages (LA) and processed to count the total number of cells within a standardised area using the ImageJ software (http://rsb.info.hih.gov/ij/docs). Statistical analyses of representative images were performed using the Student's t-test.

Figure 4: A) qRT-PCR analyses for *Pitx2c* in the left atria (LA) of *Nppa*-Cre *Pixt2*$^{-/-}$ (null) mice and control wild-type (wt) at E16.5. B) Schematic diagram showing annealing of primers used in A.

Figure 5: *In silico* analysis of the putative TSS regulatory sequences of *Isl1*, *Mef2c* and *Tbx5* genes to identify highly conserved *Pitx2*–binding sites close to binding sites for other cardiac transcription factors.

SUPPLEMENTARY METHODS

ESC Culture and Differentiation

ESCs were thawed onto and passaged on mitomycin C-inactivated mouse embryonic fibroblast (MEF) feeder layers on gelatin coated plates. Cultures were maintained in DMEM supplemented with non-essential amino acids, Hepes buffer, sodium pyruvate, L-glutamine (Gibco, Invitrogen), 15% defined fetal bovine serum (Hyclone) and 1000U/mL leukemia inhibitory factor (LIF, Chemicon). A variation of the method of Hescheler et al. 1997 was used for EB formation and differentiation. Prior to differentiation, trypsinized cultures were incubated on gelatin coated plates for 50 min to allow attachment and subsequent removal of the MEF cells. Mouse ESCs were counted, and incubated as hanging drops containing 600 cells in 20 µL medium without LIF for 7 days. Embryoid bodies (EBs) were plated onto gelatin-coated 6-cm tissue culture dishes, and the medium was changed every day.

DNA Transfections

We obtained 10 independent clones containing both PGK-Neo and αMHC-*Pitx2c*-IRES-Puromycin sequences as revealed by genotyping, of which two were used in the present study (clone 8 and 22). Both lines displayed equivalent results (data not shown), thus only clone 8 is illustrated. To isolate CM overexpressing *Pitx2c*, the same hanging drop protocol was used, and puromycin (1µg/ml) was added when EBs were plated (on day 0). Fresh media with antibiotics was added daily.

Generation of conditional tissue-specific null mutant mice

Nppa-Cre transgenic mice were backcrossed into homozygous *Pitx2*floxed mice and sacrificed at embryonic day 16.5. Similarly, embryos were PCR screened with *Pitx2*- and Cre-specific primers[10]. Cre negative embryos were selected as wild type controls ($Pitx2^{fl/fl}$)

and Cre positive embryos were subdivided into heterozygous (Cre+*Pitx2*$^{fl/-}$) and homozygous (Cre+*Pitx2*$^{-/-}$) categories.

Immunofluorescent and immunohistology staining

EBs and the differentiated outgrowing cells were fixed for 20 minutes at room temperature with 4% paraformaldehyde, and blocked at room temperature using TBSA-BSAT (10 mM Tris, 0.9% NaCl,0.02% sodium azide, 2% bovine serum albumin and 0.1%Triton-x100 detergent) followed by overnight incubation with primary antibody. Embryos were dissected, fixed overnight in freshly made 4% formaldehyde at room temperature, dehydrated in increasing graded ethanol steps and embedded in paraplast. Microtome tissue sections were cut at 10 microns and mounted on AAS-treated glass slides. For histological analyses embryo sections were processed for hematoxylin and eosin staining. The antibodies used for immunostaining included mouse anti-myosin (MF20) 1/20 (DSHB), rabbit anti-Isl1 1/50 (Abcam), goat anti-Mef2c 1/50 (Santa Cruz), rabbit anti-PHH3 (phospho-histone H3) 1/50 (Millipore), goat anti-Tbx5 1/50 (Santa Cruz), rabbit anti-desmin 1/50 (Sigma). For immunofluorescence the following fluorochrome conjugated secondary antibodies were used for visualization: anti-goat Alexa-546 (Invitrogen), anti-mouse Cy2 (Jackson Labs), anti-rabbit Tritc (Jackson Labs) at 1/100 dilution. Nuclear staining was performed using DRAQ-5^{TM} (Red Fluorescent Cell-Permeable DNA probe, Biostatus Limited). Immunofluorescent detection was carried out by confocal analyses using a Leica TCS SL confocal mycroscope (Leica LCS Version 2.0). For immunohistochemistry the sections were incubated with the corresponding secondary antibody conjugated with alkaline phosphatase (Sigma) for 90 min. Thereafter, the sections were washed in PBS and coloration was revealed by INT/BCIP solution for 20 min. Finally, the samples were counterstained with haematoxylin and mounted in DPX.

RNA Isolation, Reverse Transcriptase Reaction and Quantitative real time PCR

For each *in vitro* stage at least six beating areas were dissected, pooled and stored at -80ºC until RNA isolation. Total RNA was isolated using TriPure Isolation Reagent (Roche) according to the supplier's instructions. For conditional mutant mice, RNA isolation was performed using 10 pooled left atria dissected from E16.5 hearts; rendering two pooled RNA samples sets, one of wild type control and another of homozygous Cre+*Pitx2*$^{-/-}$ embryos.

To minimize genomic DNA contamination total RNA extracted was treated with 20 U of RNase-free DNase (Roche) for 1 hour at 37ºC and then purified using a standard phenol-chloroform extraction. 1 µg of total RNA was reverse transcribed using Superscript RNase H^{-} reverse transcriptase (Invitrogen) and a 15 mer oligo-dT primer (Promega) for 1 hour at 37ºC according to the manufacturer's protocol. As negative control, each sample was subjected to the same process without reverse transcriptase.

cDNAs were amplified using specific oligonucleotides based on NCBI sequence data. The primers used in this work were obtained from Biomedal (Sevilla, Spain). Real time PCR was performed with an iCycler PCR thermal cycler (Bio Rad) and SYBR Green detection system (BioRad). PCR reactions were performed in 0.2 mL optical tubes (Bio Rad) in a 20 µL total volume containing SYBR Green Mix (Bio Rad) and 2µL of the reverse transcribed RNA (20ng of total initial RNA*). β-actin* was used in parallel for each run as an internal control. For beating areas collected at intermediate and late stages of EB-in vitro differentiation, *NKx2.5* was used in parallel for each run as internal control. Specific primers for each gene analyzed, annealing temperature and amplicon size are shown in the Table 1. As PCR is an

exponential system, a twofold increase or reduction in expression is the result of only 1 cycle. Based on these assumptions and to minimize experimental errors, we only consider for statistical analyses the results corresponding to more than twofold differences in gene expression.

TABLE 1. Gene-specific primers, anneling temperatures, and amplicon sizes for quantitative Real-Time PCR[a]

Primers	Sequence	Annealing Temperature	Amplicon size
Pitx2c	Sense: 5'-CCTCACCCTTCTGTCACCAT-3' Antisense:5'-GCCCACATCCTCATTCTT TC-3'	60ºC	179 bp
Nkx 2.5	Sense: 5'-TTGGCGTCGGGGACTTGAAC-3' Antisense: 5'-GGTGGGTGTGAAATCCGAGGGAC-3'	62ºC	206 bp
Isl1	Sense: 5'- ACCAATTGTCCAACCACCAT -3' Antisense: 5' TCCCATCCCTAACAAAGCAC - -3'	60ºC	210 bp
Mef2c	Sense: 5'- AGAAGAAACACGGGGACTATGGG-3' Antisense: 5' GGGGTGAGTGCATAAGAGGAG-3'	60ºC	288 bp
Foxh1	Sense: 5'-CCCCAAGAGGAGGAAGAAGA-3' Antisense: 5' AGAGGTGGTCGGTAGTGTGG-3'	60ºC	165 bp
Tbx1	Sense: 5'- ATCCCATGGCCGACTACAT -3' Antisense: 5' – ACGAGTACACGTTGGCTGCT-3'	61ºC	198 bp
Gata-4	Sense: 5'-CCGAGGGTGAGCCTGTATGTAATGCC-3' Antisense: 5'-GGAGGCACCACTGGAGGGAGGG-3'	64.5ºC	182 bp
Gata-6	Sense: 5'-AGTGGCTCTGTCCCTATGACTCC-3' Antisense: 5'- GGATGTGACTTCGGCAGGGG-3'	62ºC	221 bp
Tbx2	Sense: 5'-TTCCCGTTCCACCTCTCC-3' Antisense: 5'-TAAGGACTGAAGCGCAGACG-3'	62ºC	224 bp
Tbx5	Sense: 5'-TCCCAGTACCAGTGTGAGAATGG-3' Antisense: 5'-TGCTCCGTGCTGGAACATTCC-3'	62ºC	142 bp
Nppa	Sense: 5'-CGGTGTCCAACACAGATCTG-3' Antisense: 5'-TCTCTCAGAGGTGGGTTGAC-3'	62ºC	187 bp
Cx40	Sense: 5'- CAGAGCCTGAAGAAGCCAAC -3' Antisense: 5'- ATGCGGAAAATGAACAGGAC -3'	60ºC	178 bp
c-myc	Sense: 5'- TCCTGCTACCGCACAACGC -3' Antisense: 5'- GCACCTCTTGAGGACCAGTGG -3'	63º C	177 bp
Cyclin D1	Sense: 5'-TCCTGCTACCGCACAACGC -3' Antisense: 5'- CCAGCTTCTTCCTCCACTTCCC -3'	62ºC	172 bp

Cyclin D2	Sense: 5'- CTGGCCAAGATCACCCAC -3' Antisense: 5'-CACGTCTGTAGGGGTGGTG -3'	58ºC	162 bp
αMHC	Sense: 5'-CTCAGCCAGGCCAATAGAAT-3' Antisense: 5'-GACTCCATCTTCTTCTTCTGG-3'	58.2ºC	331 pb
βMHC	Sense: 5'-AGATCGCCCTCAAGGGTGGC-3' Antisense: 5'-AGGTCCTGGAGCCGCAGTAGG-3'	62ºC	196 pb
MLC2v	Sense: 5'-TGTTCCTCACGATGTTTGGG-3' Antisense: 5'-CTCAGTCCTTCTCTTCTCCG-3'	58ºC	291 pb
MLC2a	Sense : 5'-AAGCCATCCTGAGTGCCTTCCG-3' Antisense: 5'-GGTGTCAGCGCAAACAGTTGC-3'	62ºC	139 pb
β-actin	*Sense:* 5´-TGA GGA GCA CCC TGT GCT-3´ *Antisense*5´-CCA GAG GCA TAC AGG GAC-3´	62ºC	143 bp
E-cadherin	*Sense:* 5' - CCT GCC AAT CCT GAT GAA AT - 3' *Antisense:* 5' - GTC CTG ATC CGA CTC AGA GG - 3'	60ºC	164 bp
Nestin	*Sense:* 5' - AGG CTG AGA ACT CTC GCT TG - 3' *Antisense:* 5' - AGA GAA GGA TGT TGG GCT GA - 3'	60ºC	152 bp
MyoD	*Sense:* 5' - GGC TAC GAC ACC GCC TAC TA - 3' *Antisense:* 5' - TCC CTG TTC TGT GTC GCT TA- 3'	60ºC	204 bp

[a]PCR, polymerase chain reaction

Each PCR reaction was performed in triplicate and repeated at least in three distinct biological samples to obtain a representative average. The expression of each gene was normalized to *β-actin*. The relative levels of expression for the genes analyzed in beating areas overexpressing *Pitx2c* were also calculated as the ratio of the expression of each gene and the cardiac-specific transcription factor *Nkx2.5*. PCR products were analyzed by 2% agarose gel electrophoresis to verify their size. For statistical analyses of data sets the unpaired Student's t-tests were used. Significance levels or p values are stated in each corresponding figure legend.